STUDIES ON THE CHEMOAUTOTROPHIC IRON BACTERIUM *FERROBACILLUS FERROOXIDANS*

I. An Improved Medium and a Harvesting Procedure for Securing High Cell Yields

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Studies of the biochemistry and physiology of the obligate chemolithoautotrophs have been largely restricted to the genus *Thiobacillus* (Vishniac and Santer, 1957) and the nitrifying bacteria (Fry and Peel, 1954; Lees, 1955; Engel and Alexander, 1958). Such studies were made possible by the prior development of media and methods that would yield large numbers of physiologically active cells. Unfortunately, parallel developments with the obligate autotrophic iron bacteria have not proceeded at similar rates.

Interest in the iron bacteria has remained high ever since Winogradsky first postulated that the energy derived from the oxidation of ferrous iron could serve as the sole support for the growth of a CO_2-assimilating microorganism. Since then, a variety of species of iron bacteria has been described in the literature, where it appears that the only requisite for this appellation is the ability to precipitate oxidized iron in sheaths, stalks, capsules, or the surrounding medium. But, it is doubtful whether iron oxidation by most of these organisms contributes materially to the energy supply needed for growth, i. e., they are heterotrophs (Pringsheim, 1949*a*, *b*; Stokes, 1954).

To our knowledge, only a few species are presently considered to be obligate chemoautotrophs; namely *Gallionella* spp., *Thiobacillus ferrooxidans*, and *Ferrobacillus ferrooxidans*. The recent work of Kucera and Wolfe (1957) with *Gallionella ferruginea* has demonstrated the difficulty of obtaining suitable yields of this organism in pure culture. Further, their photographs indicate that separation of cells from the tangled mass of inert stalks would indeed be a formidable task. The validity of the species *T. ferrooxidans* has been questioned (Leathen *et al.*, 1953). These authors believe the ability to oxidize thiosulfate and iron may not be due to a single species but to either a purely chemical reaction causing decomposition of the thiosulfate, or to the combined activities of *T. thiooxidans* and *F. ferrooxidans*. The latter species, in addition to being an obligate chemoautotroph, possesses the advantage of being devoid of any sheath, stalk, or capsule impregnated with iron. Therefore, it was deemed advisable to concentrate all efforts toward developing a medium capable of supporting large numbers of physiologically active cells of *F. ferrooxidans*. The present report concerns the development of such a medium, a method of harvesting cells virtually free of precipitated iron, and some observations on factors affecting growth.

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MATERIALS AND METHODS

F. ferrooxidans strain TM was kindly supplied by W. W. Leathen. When received, this strain had undergone 23 serial passages on the ferrous iron-silica gel medium of Leathen *et al.* (1956) and had been maintained by monthly serial transfer in their inorganic ferrous iron liquid medium for several years.

We have confirmed the autotrophic growth of *F. ferrooxidans* in these authors' liquid medium, young cultures oxidizing the 200 ppm ferrous iron in 3 to 4 days when incubated at 28 C in shake culture. However, in terms of cell numbers, growth was sparse. With the aim of increasing cell numbers, the liquid medium of Leathen *et al.* (1956) was modified quantitatively until medium 9K was developed (table 1). The basal salts and iron solution were autoclaved separately and combined when cool. Some oxidation of iron occurred during autoclaving but the loss of ferrous iron was not appreciable. The modified medium (9K) could be stored for at least two

weeks at refrigerator temperature without noticeable oxidation. The medium exhibited a precipitate (probably ferrous and ferric phosphates), was opalescent and green, had a pH of 3.0 to 3.6, and contained 9000 ppm ferrous iron. The culture has been maintained for more than a year by transferring twice a week into 100 ml of modified medium dispensed in 250 ml Erlenmeyer flasks which were incubated at 28 C on a reciprocal shaker.

For large scale growth, cells were grown both at room temperature and at 28 C in submerged culture under forced aeration in a 12-L pyrex-glass carboy containing 10 L of medium. Air, at the rate of approximately 0.6 L per min per L medium, was sterilized by serial passage through a Kelly bottle packed with sterile glass wool and a humidifier of sterile distilled water fitted with a Selas porcelain filter candle (porosity 10), then dispersed in the medium through a second filter candle. A 10 per cent inoculum was added to the carboy from a 1-L culture which was prepared by inoculating 1 L of medium with 100 ml of a 2-day old culture and incubating at 28 C for 24 hr with forced aeration.

To secure cell suspensions containing a minimum of precipitated iron, the following harvesting procedure was devised. The contents of the carboy were centrifuged in a Sharples supercentrifuge at the rate of 2 to 3 L per hr. The resulting paste of cells and precipitate was suspended in approximately 300 ml cold distilled water (acidified to pH 2.6 with H_2SO_4), shaken vigorously in a 1-L reagent bottle for roughly 1 min, and allowed to stand in the refrigerator for at least 6 hr. The turbid supernatant fluid was carefully removed from the underlying layer of precipitated iron; this procedure was repeated twice. The cells, contained in the combined supernatant fluids, were removed by centrifugation in a Servall angle centrifuge (type XL), washed 3 times in distilled water (pH 2.6), and brought to a volume of 50 ml with distilled water (acidified to pH 3.5 with H_2SO_4). The "clean suspensions" were stored in the refrigerator and remained viable for at least 1 month. The bacterial nitrogen of these suspensions was determined by the micro-Kjeldahl technique. Cell numbers, during growth and in the clean suspensions, were determined by direct counts employing a Petroff-Hausser bacterial counting chamber. The level of ferrous iron was determined volumetrically with 0.01 N $K_2Cr_2O_7$ according to the method of Pierce and Haenisch (1950), except for the use of the decolorizing solution of Mellon (1956) and partially oxidized sodium diphenylamine sulfonic acid indicator (Willard and Young, 1933).

TABLE 1

Comparison of media for growth of Ferrobacillus ferrooxidans

Components	Leathen *et al.* (1956)	Modified Medium (9K)
	g	g
Basal salts:		
$(NH_4)_2SO_4$	0.15	3.0
KCl	0.05	0.10
K_2HPO_4	0.05	0.50
$MgSO_4 \cdot 7H_2O$	0.50	0.50
$Ca(NO_3)_2$	0.01	0.01
Distilled H_2O	1000 ml	to 700 ml
10 N H_2SO_4	—	1.0 ml
Energy source:		
$FeSO_4 \cdot 7H_2O$	10 ml of a 10% (w/v) solution	300 ml of a 14.74% (w/v) solution

RESULTS

Results of preliminary growth studies with *F. ferrooxidans* showed the optimum temperature to be 28 C. This temperature was used in all subsequent studies. No growth occurred at 37 C.

Medium 9K readily supported the growth of *F. ferrooxidans*; cell counts ranged from 2 to 4×10^8 cells per ml as opposed to only 7×10^6 cells per ml in the medium of Leathen *et al.* (1956). Typical growth curves comparing the two media are illustrated in figure 1. Rapid growth required sufficient aeration to insure an adequate supply of O_2 and CO_2. Figure 2 illustrates the difference in rates of growth for stationary and shake cultures. Apparently, sufficient O_2 and CO_2 were dissolved in the medium to support growth of at least 3×10^7 cells per ml in stationary culture. Thereafter, the growth rate fell off rapidly but was rejuvenated once proper aeration was supplied.

F. ferrooxidans depends upon ferrous iron as its sole energy source; once all the iron was oxidized growth ceased. In addition, the rate of growth closely paralleled the rate of substrate utilization as shown in figure 3. Generation times of

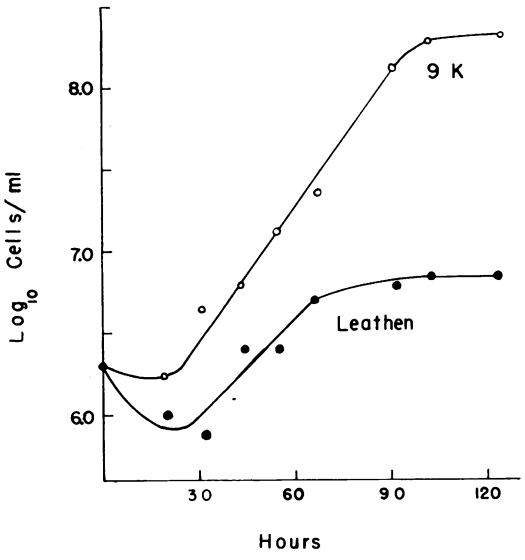

Figure 1. Comparison of the growth of *Ferrobacillus ferrooxidans* in the modified medium (*9K*) and in the medium of Leathen *et al.* (1956).

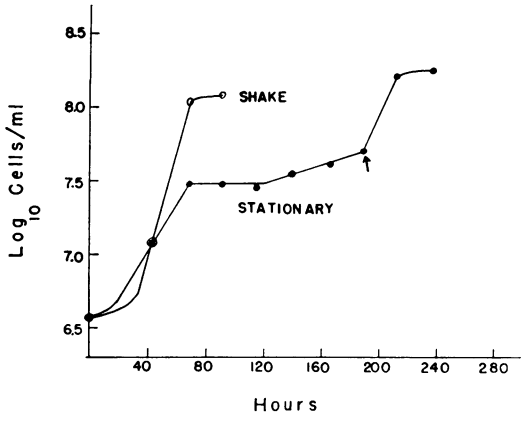

Figure 2. Comparison of the growth of *Ferrobacillus ferrooxidans* in the modified medium in shake and stationary culture. At the time indicated by the *arrow*, the stationary culture was placed on the shaker.

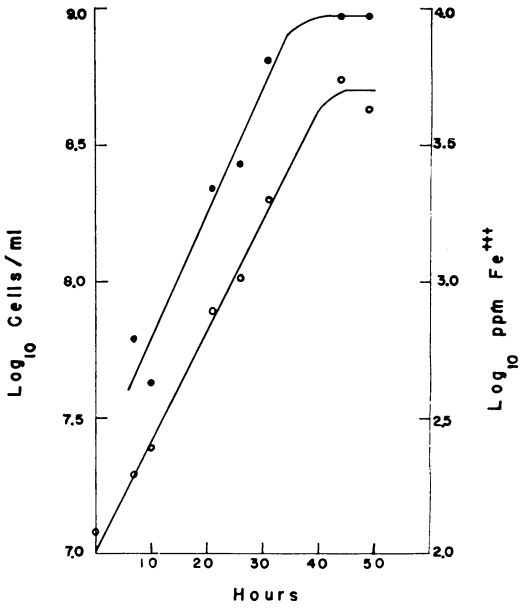

Figure 3. The rate of growth of *Ferrobacillus ferrooxidans* as a function of substrate utilization. Cells were grown in 10 L modified medium in a 12-L carboy with forced aeration. Only ferrous iron was measured. The initial ferric iron concentration was taken to be zero. Thereafter, the difference between the initial and subsequent ferrous iron determinations was taken to be the ferric iron concentration. Plotting the ferric rather than ferrous iron concentration gives a curve with a positive slope that more graphically portrays the parallelism between growth and substrate utilization. *Solid circles*, iron; *open circles*, cells.

cultures, whether aerated by shaking or with forced aeration, ranged from 5.3 to 9.6 hr; the average value was approximately 7.0 hr.

Ferrous iron at a concentration of 9000 ppm appeared to be optimum for the most rapid production of high yields of cells. Progressive increases in the initial Fe^{++} concentration in medium 9K failed to increase final cell yields and progressively decreased growth rates. This is shown in figure 4. No stimulation of growth occurred when a trace element solution consisting of Mn^{++}, Cu^{++}, B^{+++}, Zn^{++}, Mo^{++++++}, Co^{++} was added to the basal salts of the modified medium. Similarly, the substitution of tap water for distilled water had no effect. *F. ferrooxidans* probably received any required trace element (s) as impurities in the salts, iron, or distilled water. Increasing the concentration of potassium, nitrate, ammonium, or magnesium over the levels present in the basal salts of medium 9K had no effect on growth. However, increasing the concentration of dibasic potassium phosphate to 2.75 g per L (or more) resulted in complete inhibition of growth.

The medium became progressively more acid in reaction as growth proceeded, for the medium is poorly buffered in the pH range 2.0 to 4.5. The

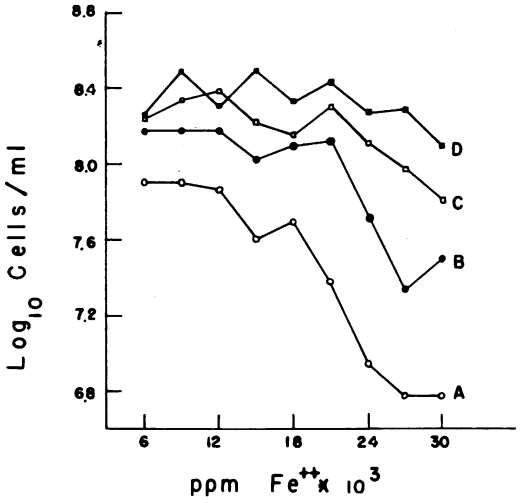

Figure 4. The effect of initial iron concentration on the growth of *Ferrobacillus ferrooxidans* in the basal salts of the modified medium. Curves *A*, *B*, *C*, and *D* represent total growth in 2, 3, 4, and 5 days, respectively.

drop in pH is illustrated in figure 5. Attempts to stabilize the pH at 3.6 by the incorporation of phthalate, citrate, and citrate-phosphate buffers were unsuccessful since all of them proved to be toxic at concentrations ranging from 0.02 to 0.50 M. The two citrate buffers, however, permitted growth at 0.01 and 0.015 M (the latter only after an 8-day lag) but the buffer capacity was too low to control the pH. Phthalate buffer was completely inhibitory at concentrations of 0.01 and 0.015 M. The periodic addition to the medium of sufficient 5 per cent sodium carbonate to return the pH to 3.6 had no effect on growth, while the initial incorporation in the medium of tricalcium phosphate (0.25 g/100 ml medium) had little effect upon growth. The addition to the medium of calcium carbonate (0.25 to 1.0 g/100 ml medium) had no effect on growth up to the level of 0.50 g but severely limited growth at higher concentrations.

The harvesting efficiency of cultures grown in large batches was consistently between 40 and 65 per cent. Thick cell suspensions (final volume 50 ml) were obtained which contained as high as 2.6×10^{10} cells per ml, and for every 1.0×10^{10} cells there was 0.191 mg bacterial N present. Heavy clean suspensions were cream to tan in color depending upon the amount of residual iron present.

Attempts to estimate cell numbers were unsuccessful employing silica gel plates. No gelation occurred using various combinations of silica, basal salts, and ferrous iron. Also, the use of washed or unwashed agar to solidify media was abandoned because any colonies that developed after one week incubation were so small as to be indistinguishable from air bubbles or other minute surface irregularities when examined by both transmitted and reflected light using 31 × magnification. In addition, considerable atmospheric oxidation of surface ferrous iron further obscured the recognition of colonies. In any case, colonies were extremely small. Negative staining of impression smears revealed that those colonies present contained no more than 50 cells per colony. Turbidimetric estimations of growth were precluded by the presence of the inorganic precipitate. These observations led to the use of direct counts as the most rapid and reliable method of estimating cell numbers.

We have confirmed the observations of Leathen *et al.* (1956) on the morphology of *F. ferrooxidans*. The cells are gram-negative rods and were difficult to stain. Smears of clean cell suspensions either did not stain, or stained weakly with carbol fuchsin, safranin, malachite green, crystal violet, methylene blue, bismarck brown, and aniline blue. Smears were stained, however, either by steaming for 5 min in carbol fuchsin, or by employing the silver-plating staining method of Rhodes (1958). No flagella were demonstrated with the latter staining method. Negative staining of smears with congo red was accomplished

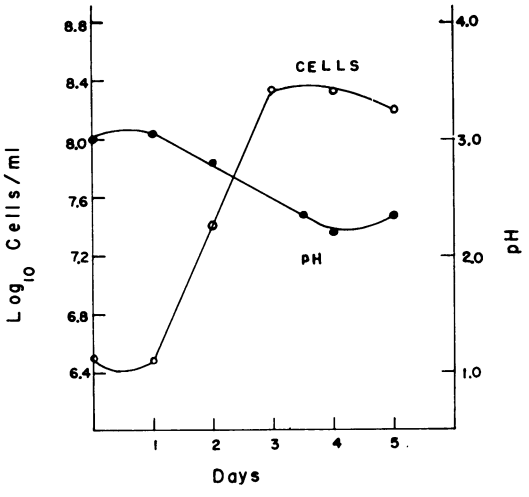

Figure 5. Changes in pH of the modified medium during the growth of *Ferrobacillus ferrooxidans*.

providing the iron content of the smear was negligible, as was the case with clean cell suspensions. However, when smears prepared from cells in medium 9K were stained with congo red, a blue-black precipitate formed that completely obscured the cells. This precipitation was avoided by washing the heat-fixed smear in tap water for about 30 sec before applying the congo red.

F. ferrooxidans has been described as actively motile (Leathen *et al.*, 1956). We have never observed active motility when cells were grown in the medium of Leathen *et al.* or in medium 9K. However, we have observed some evidence of weak motility during counting. Usually, some cells were seen to adhere by one pole to either the upper or lower surface of the counting chamber while describing a rotary motion at the unattached pole.

DISCUSSION

Estimations of the growth of the autotrophic iron bacteria have usually been reported as the rate of oxidation of substrate (Bryner and Jameson, 1958; Temple and Colmer, 1951; Leathen *et al.*, 1956). Beck and Elsden (1958) described a liquid medium that supported the growth of an iron bacterium "probably identical to *Ferrobacillus ferrooxidans*" with a cell density of 3×10^7 cells per ml in the stationary phase of growth. In comparison, medium 9K supported at least 2×10^8 cells per ml in the logarithmic phase and 4 to 5 $\times 10^8$ cells per ml in the stationary phase.

F. ferrooxidans has previously been shown to be autotrophic in character, not utilizing any of the usual organic bacteriological media (Leathen *et al.*, 1956). The present demonstration of the parallel rates of iron oxidation and cell growth, the cessation of growth upon substrate exhaustion, and the fact that increasing the iron content of the medium of Leathen *et al.* (as in medium 9K) results in markedly increased cell numbers, are further strong evidence of the autotrophic nature of this organism.

Medium 9K contains two oxidizable constituents, ammonium and ferrous iron. It might be argued that ammonium is being used as an energy source for growth. However, the evidence cited in the preceding paragraph would indicate that this is probably not the case.

The increase in acidity during growth is probably a reflection of reactions 1 and 2.

$$4FeSO_4 + 2H_2SO_4 + O_2 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O \quad (1)$$

$$2Fe_2(SO_4)_3 + 12H_2O \rightarrow 4Fe(OH)_3 + 6H_2SO_4 \quad (2)$$

$$\text{Sum: } 4FeSO_4 + O_2 + 10H_2O \rightarrow 4Fe(OH)_3 + 4H_2SO_4$$

Reaction 1 is catalyzed by *F. ferrooxidans* as the means of obtaining its energy. During this reaction acid is removed and one would expect the medium to become more basic. But hydrolysis takes place (reaction 2) producing more acid than is consumed in reaction 1 so that the net effect is an increase in the acid content of the medium.

Our knowledge of iron metabolism is less than complete. Iron is known to be a component of certain respiratory enzymes, haem-containing compounds, the iron storage compound ferritin, ferrichrome (Neilands, 1952), and ferrichrome A (Garibaldi and Neilands, 1955). Additional iron compounds of biological interest are largely unknown; due, in part, to the dearth of biological systems utilizing iron in greater than trace amounts. The study of organisms with a greatly exaggerated iron metabolism would be of distinct advantage. Obviously, *F. ferrooxidans* possesses this attribute. As an obligate chemoautotroph it must transport reduced iron into the cell, oxidize it to the ferric state, and transport it out of the cell. In addition, the low energy yield (11.3 k cal/g-atom) of the reaction:

$$Fe^{++} \rightarrow Fe^{+++} + e$$

requires that relatively enormous quantities of iron must be processed (e. g., the utilization of all the ferrous iron in 1 L of medium 9K means the oxidation of 9 g of iron has taken place). Since *F. ferrooxidans* can now be propagated in large numbers and, what is equally important, in relatively clean condition, this organism should prove a valuable tool for supplying some of the missing parts in the over-all picture of iron metabolism.

SUMMARY

An improved medium for the obligate chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans* that will support the growth of 2 to 4×10^8 cells per ml is described, together with a method of harvesting cells relatively free from

the accompanying precipitate of oxidized iron. The demonstration of the coincidence of ferrous iron oxidation and growth further confirms the autotrophy of this organism. Some observations concerning the nutrition and morphology of *F. ferrooxidans* are presented.

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